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Review

Tumor and its microenvironment: A synergistic interplay

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ABSTRACT

The mutual and interdependent interaction between tumor and its microenvironment is a crucial topic in cancer research. Recently, it was reported that targeting stromal events could improve efficacies of current therapeutics and prevent metastatic spreading. Tumor microenvironment is a "complex network" of different cell types, soluble factors, signaling molecules and extracellular matrix components, which orchestrate the fate of tumor progression. As by definition, cancer stem cells (CSCs) are proposed to be the unique cell type able to maintain tumor mass and survive outside the primary tumor at metastatic sites. Being exposed to environmental stressors, including reactive oxygen species (ROS), CSCs have developed a GSH-dependent antioxidant system to improve ROS defense capability and acquire a malignant phenotype. Nevertheless, tumor progression is dependent on extracellular matrix remodeling, fibroblasts and macrophages activation in response to oxidative stress, as well as epithelial mesenchymal transition (EMT)-inducing signals and endothelial and perivascular cells recruitment. Besides providing a survival advantage by inducing *de novo* angiogenesis, tumor-associated vessels contribute to successful dissemination by facilitating tumor cells entry into the circulatory system and driving the formation of pre-metastatic niche. In this review, we focus on the synergistic effect of hypoxia inducible factors (HIFs) and vascular endothelial growth factors (VEGFs) in the successful outgrowth of metastasis, integrating therefore many of the emerging models and theories in the field.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and one of the major causes of death worldwide [1]. The prevention and the early diagnosis are surely the most important approaches for reducing the burden of CRC, given the symptoms of early disease occur just in 5% of cases. A significant portion of patients who receive surgery and adjuvant therapy still develop recurrences and metastasis and this phenomenon seems to be driven in some cell subsets by the acquisition of resistance to conventional therapy, such as chemo- and radio-therapy [2].

Growing evidence indicates that a cellular subpopulation with stem cell like features, commonly referred to as cancer stem cells (CSCs), is critical for tumor generation and maintenance.

A recent study showed that within the tumor population it is possible to identify a heterogeneous population of cells with different biological roles [3]. Recent advances in stem cell biology are revealing that this cellular fraction shares many properties with normal adult stem cells, including dormancy (quiescence), active DNA repair machinery, the expression of several ABC drugs transporters and an intrinsic resistance to apoptosis [4]. As their normal counterpart, the colon CSCs reside in a specialized microarchitectonic structures or niches that respond to both local and systemic conditions providing also protection against conventional therapies [5].

Moreover, microenvironmental stimuli, such as those involved in the epithelial-mesenchymal transition (EMT) and hypoxia, indirectly contribute to chemoresistance by inducing in cancer cells a stem like-phenotype. Understanding the driving force of tumor progression and the relationship between cancer cells and microenvironment could be fundamental in developing innovative therapeutic strategies for a better and definitive response on patient treatments.

2. CRC, stem cell niche and colon CSCs

It is widely accepted that CRC progression is driven by the acquisition of 4–5 progressive mutations in oncogenes or tumor

Abbreviations: CSCs, cancer stem cells; CRC, colorectal cancer; EMT, epithelial mesenchymal transition; ECM, extracellular matrix; ROS, reactive oxygen species; MMPs, matrix metalloproteinase; CAFs, cancer-associated fibroblasts; CAMs, cancer-associated macrophages; GSH, reduced glutathione; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

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suppressor genes [6]. Some driver mutations frequently occur in the same gene sequences and are shared by most of the people affected by this cancer, whereas some mutations are different and responsible of the final cancer phenotype in individual patients [7]. Most of the information about CRC derives from the study of familial adenomatous polyposis (FAP), an autosomal dominant colon cancer syndrome caused by *APC* gene mutation [8]. APC is involved in the regulation of Wnt pathway that, as we will discuss later in this review, can regulate cell proliferation, differentiation, migration and apoptosis [9]. Tumor progression is also achieved by other mutations such as *KRAS*, *SMAD2/4*, *TP53* and deletion of chromosome 18q [10].

It was recently demonstrated that despite the great heterogeneity and biological diversity of CRC it is possible to distinguish three different subtypes. De Sousa et al. indeed showed that two of these subtypes have already been identified for chromosomal-instable and microsatellite-instable cancer. A third one, prognostically unfavorable, is characterized by microsatellite stability and relatively more CpG island methylator phenotype-positive, thus rendering it impossible to be identified on the basis of characteristic mutations [11].

The presence of a distinct population with stem cell characteristics among disseminated and circulating cancer cells may be of clinical relevance, not only for their putative role in metastasis formation and recurrence, but also for their role in resistance to conventional therapy. CSCs are likely to share many properties of normal stem cells as mentioned above, which may underlie their capacity to survive therapeutic protocols based on genotoxic agents targeting actively proliferating cells [12].

First invoked by Paget, the "seed and soil" hypothesis suggests that the successful growth of metastatic cells depends on the interactions and properties of cancer cells (seeds) and their potential target organs (soil). Additionally, new concepts include: (i) the role of cancer stem-like cells as putative cells of metastatic origin (the "seeds"); (ii) the mechanism of EMT in driving epithelial cell into the blood stream to avoid *anoikis*, or anchorage independent cell death; and (iii) the reverse process of EMT, or mesenchymal to epithelial transition (MET), which promotes conversion back to the parent cell morphology and growth of macrometastasis in the target organ, open a new broad of aspect on this issue [13].

The microenvironment plays a crucial role in maintaining the pluripotency of colon SCs at the base of colon crypts influenced by fibroblast, endothelium and inflammatory cells, cytokines and growth factors secreted by these cells (in particular HGF) thus finely regulating the balance between self-renewal and differentiation of the staminal population [14–16]. The most characterized pathway involved in the maintenance of colon stem cells is Wnt [17–19], and it is clearly highlighted by the different expression of Wnt members along the colon crypt [20], even if the maintaining of stemness and the differentiation pattern is actually the result of the fine collaboration with other important pathways, such as PTEN-PI3K-Akt [21,22], BMP [23], Notch [24] and Sonic hedgehog (Shh) [25].

3. EMT, pre-metastatic niche and metastasis formation

Metastasis formation is considered a complex multi-step process with sequential molecular and cellular events that permit transformed cells to gain access to the blood stream (intravasation), survive their journey through the blood stream, and ultimately traverse through the microvasculature of target organs (extravasation) to deposit, survive, and grow in a foreign tissue environment. The EMT represents the first step of this highly regulated cascade and it is an important biological process initially studied in normal tissues during the organogenesis and then extended in the pathogenesis of cancer diseases, particularly referred to the acquisition of migratory phenotype in CRC cells [26]. After extravasation from the circulation into the target organ, aberrant cells must implant, proliferate, and induce angiogenesis in order to survive and grow in a foreign and presumably "hostile" environment. These phenomena are driven not only by genetic and/or epigenetic alteration of cancer cells, but also by the non-neoplastic stromal cells [27].

The EMT is characterized by the loss of epithelial properties, including the apico-basal polarity and cell adhesion, the E-cadherin, occluding and cytokeratins expression, and at the same time the acquisition of N-cadherin, vimentin, fibronectin, Twist1, zinc-finger proteins (SNAIL, SLUG, ZEB) and matrix metalloproteinases (MMPs) expression, all events that lead to an increased cell mobility [28]. Moreover, EMT-inducing factors released by the surrounding microenvironment [29] can affect the invasive phenotype in epithelial malignancies initiation. Key regulators of this process are TGF-β (by the activation of Twist, SLUG and ZEB2), PI3K/Akt (increasing the mTOR kinase expression), Shh and Wnt [30,31].

Currently, dissemination and spread of cancer cells during the tumor progression are elective events underling the invasion through the tissue extracellular matrix (ECM). It was recently shown that tumor cells have two different modes of motility: (1) the acquisition of a mesenchymal phenotype, as previously described that identifies a mesenchymal motility mode and (2) the amoeboid migration [32]. The mesenchymal mode is characterized by the acquisition of an elongated morphology and activation of the small GTPase Rac [33]; the amoeboid motility is defined by a rounded or ellipsoid cell morphology and weak interactions with the surrounding matrix, driven by Rho expression, which induce membrane blebbing through Rho-associated protein kinase (ROCK)-dependent myosin II phosphorylation and consequent actomyosin contractility [34]. These two migration modes are interconvertible and regulated by microenvironmental influences. The possibility to switch from one mode to the other one highlights the cell plasticity that accomplishes movement from the primary tumor, establishment in an ectopic site, and survival therein [35].

The balance between high levels of activated Rac and Rho proteins regulates finely the motility mode. Moreover, Rac signaling inhibits amoeboid movement through its effector WASP-family verprolin-homologous protein 2 (WAVE2), and in the same way Rho/ROCK suppresses Rac by the activation of ARHGAP22, a GTPase-activating protein (GAP) [36].

Although *RHO* gene mutations are extremely rare, their altered expression has been assessed in many human cancers, including CRC. In particular, RhoA is frequently overexpressed and its induction is rapidly mediated by TGF-β [37], while depletion of Rac1 strongly correlates with the inhibition of lamellipodia formation, cell migration and invasion in carcinoma cells [38].

Furthermore, recent study established the independent contribution of *KRAS* and *BRAF* mutations, which rarely coexist in human tumors, to migration and invasion of CRC cells through Rho GTPases signaling. Although KRAS and BRAF are common members of the same pathway, Makrodouli et al. showed that *BRAF* mutation enhances cell migration through RhoA activation, and its effect is more pronounced compared to KRAS. These findings are expected to eventually result in tailor-made therapies against Rho pathway components, since it depends on the genetic background of the cancer patient [39].

4. Status redox and hypoxia: two sides of the same coin

In the absence of an aberrant microenvironmental stimuli, genetic and epigenetic alterations in tumor cells are insufficient to induce primary tumor progression [27]. Either through structure and function-based mechanisms, including ECM remodeling,

release of cytokines and growth factors, metabolic changes, or activation of stromal components, microenvironment enables tumor cells to achieve an aggressive phenotype [32].

As observed, reactive oxygen species (ROS) have emerged as an important factor affecting several cancer hallmarks. ROS are involved in the acquisition of self-sufficiency in proliferation signals by a ligand-independent receptor tyrosine kinase transactivation as well as loss of contact inhibition and anchorage-dependence cell growth. The development of a more aggressive phenotype is also promoted by ROS through MMPs secretion, EMT program activation, Met overexpression and regulation of cellular plasticity induced by the Rac1/RhoA antagonism [40,41]. Moreover, ROS sustain *de novo* angiogenesis by inducing the recruitment of perivascular cells and the activation of endothelial progenitors through the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) release. Besides being involved in evading apoptosis by the activation of survival pathways, specifically PI3K/AKT, NF-kB, and *anoikis* resistance, ROS increase the sensibility to mutagenic agents and help escape from the immune surveillance system [42].

Oxidative stress can derive from either extrinsic or intrinsic source (Fig. 1). Cancer-associated-fibroblasts (CAFs) or -macrophages (CAMs) synergize in the induction of a pro-oxidant environment. Due to the activation of Nitric Oxide Synthase 2 (NOX2), CAMs can directly produce ROS resulting in CAFs recruitment and MMPs activation [43]. Moreover, by secreting the master pro-inflammatory cytokine TNFα, CAMs prime the NF-kB activation in both stromal and cancer cells, which in turn up-regulates *SNAI1* expression [44]. In response to intrinsic and extrinsic oxidative stress, CAFs support tumor growth and promote EMT changes in cancer cells by secreting growth factors and ECM degrading proteases. Moreover, their production of extracellular matrix proteins promotes the recruitment of endothelial precursor cells from bone marrow [45]. Aging-induced oxidative stress concurs to transform fibroblasts into pro-inflammatory cells and induce an EMT program in the neighboring epithelial cells by secreting the so-called senescent activated secretory pathways (SASP) factors, which include pro-inflammatory cytokines and MMPs [46]. Klimova et al. demonstrated that hypoxia also improves ROS generation by deregulation of the mitochondrial complex III resulting in ROS release into the cytosol [47].

Interestingly, TGFβ has been correlated to redox control of EMT, either directly by the activation of MAPK or indirectly by ERK-mediated Smad 2 phosphorylation. As shown by Rhyu et al., in renal tubular epithelial cells, TGFβ1 stimulation induces E-cadherin loss, α-SMA and fibronectin up-regulation. These EMT-related molecular events are prevented by the inhibition of both NADPH oxidase (NOXes) and mitochondrial electron transfer chain subunit I, suggesting that NOXes and mitochondrial metabolism are important sources of TGFβ-induced cellular ROS [48]. Similarly, Zhang et al. identified ferritin heavy chain (FHC) as a critical modulator of TGFβ-induced EMT. By repressing the synthesis of FHC, a cellular iron storage protein, TGFβ promotes iron release and subsequent increase in the intracellular labile iron pool (LIP), which is associated with redox-mediated activation of p38MAPK. Thus, FHC overexpression abrogates TGFβ-induced LIP increase resulting in ROS elimination and EMT suppression [49].

Cancer cells exacerbate the oxidant microenvironment by enhanced basal metabolic activity through aberrant growth factors and cytokines signaling as well as increased activity of ROS-producing enzymes, such as NOXes, cyclooxygenase (COXes) or lipoxygenases (LOXes) [50]. Moreover, high levels of ROS may result from down-regulation of Jun D, a transcriptional activator of FHC that is known to minimize LIP-dependent ROS generation [51].

To protect themselves from oxidative stress, cancer cells develop adaptation strategies, including increased expression of

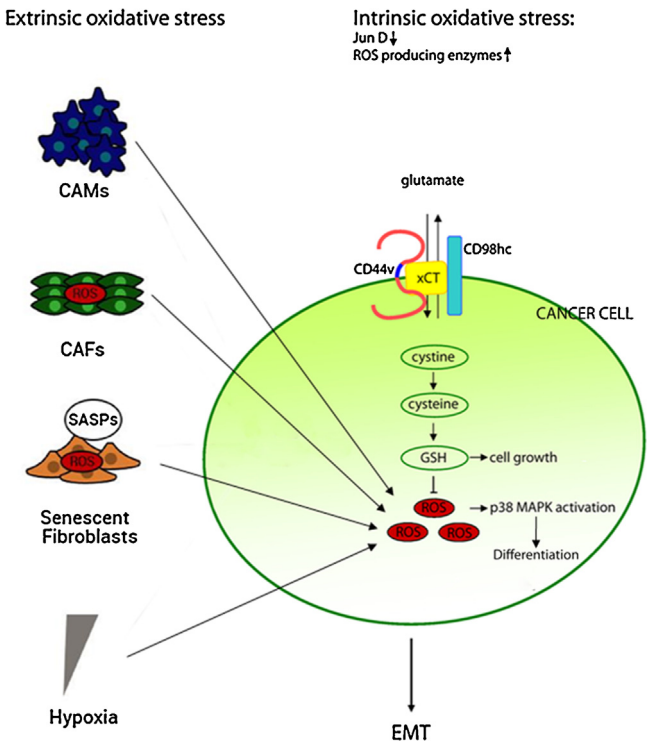

Fig. 1. Extrinsic and intrinsic production of oxidative stress. CAMs and hypoxia induce a pro-oxidant environment, mandatory for CAF activation and senescent fibroblasts conversion into pro-inflammatory cells, affecting in turn EMT of cancer cells. Due to Jun D downregulation and increased activity of ROS-producing enzymes, cancer cells exacerbate the production of oxygen radicals. CD44v stabilizes the subunit xCT at the plasma membrane by promoting GSH synthesis and tumor growth. Cancer-associated macrophages (CAMs), cancer-associated fibroblasts (CAFs), senescent activated secretory pathways (SASPs), reactive oxygen species (ROS), reduced glutathione (GSH), CD44 variant (CD44v), the light-chain subunit of cystine–glutamate antiporter system xc(-) (xCT), epithelial mesenchymal transition (EMT).

scavenger anti-oxidative enzymes and pro-survival molecules. Particularly, reduced glutathione (GSH) is the major intracellular antioxidant factor by reducing the ROS levels and suppressing ROS-dependent activation of p38MAPK. Ishimoto et al. demonstrated that in gastrointestinal cancer cells a CD44 variant (CD44v) maintains high levels of GSH by stabilizing the xCT expression at the plasma membrane. xCT is the light-chain subunit of cystine–glutamate antiporter system xc(-), which exchanges extracellular cystine uptake for intracellular glutamate, thereby promoting GSH synthesis (Fig. 1). At first, glutamate–cysteine ligase couples glutamate and cysteine to form γ-glutamylcysteine. Glutatione synthetase then catalyzes the formation of GSH from glycine and γ-glutamylcysteine. Since cysteine availability is a rate-limiting factor for GSH synthesis, CD44-mediated stabilization of xCT plays a key role in the GSH-dependent antioxidant system, promoting the proliferation of cancer cells and the formation of lethal gastrointestinal tumors. This is supported by the observation that CD44 depletion reduces the number of proliferating tumor progenitor cells and inhibits gastric tumor development in Gan (Gastric Neoplasia) mice through the ROS-dependent p38MAPK activation and $p21^{CIP1/WAF1}$ upregulation. The antioxidant potential of gastric cancer cells confers resistance to ROS-inducing anticancer drugs, such as cisplatin and docetaxel. Consistently, in an HCT116 xenograft model, the specific xCT inhibitor sulfasalazine suppresses CD44-dependent tumor growth in parallel with the activation of p38MAPK, suggesting that the suppression of xCT by sulfasalazine might impair the ROS defense ability of CD44v-expressing CSCs and improve the efficacy of currently available treatments [52] (Fig. 2).

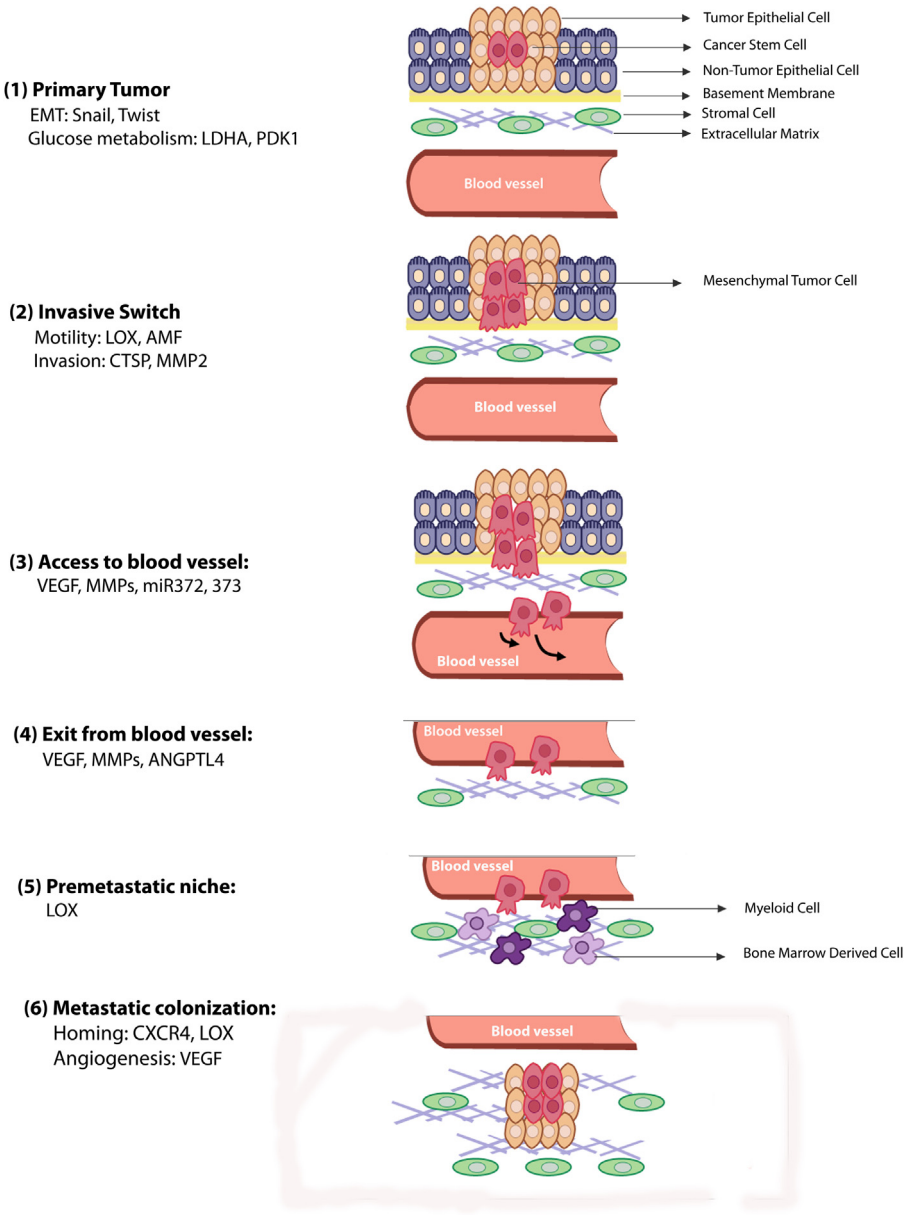

Fig. 2. Regulatory functions of hypoxia in different steps of metastasis. (1) During primary tumor growth, hypoxia acts as inductor of "glycolytic" phenotype and executor of EMT. (2) Under hypoxia, tumor cells gain an improvement in motility and invasion capacity, facilitating thereby detachment and dissemination from the primary site. (3 and 4) Increased expression of VEGF and MMPs induced by hypoxia is critical to penetrate the vasculature and promote the subsequent exit. (5) By the recruitment of bone marrow-derived cells and $CD11b^{+}$ myeloid cells to secondary organs, LOX secreted by hypoxic tumor cells forms the premetastatic niche. (6) Hypoxia-dependent induction of CXCR4 and angiogenesis contribute to the successful metastatic colonization. Epithelial mesenchymal transition (EMT), lactate dehydrogenase A (LDHA), pyruvate dehydrogenase kinase 1 (PDK1), Lysyl oxidase (LOX), autocrine motility factor (AMF), cathepsin D (CTSD), matrix metalloproteinase (MMPs), vascular endothelial growth factor (VEGF), angiopoietin-like 4 (ANGPTL4).

CD44 and its variant isoforms have already been identified as tumor metastasis-associated proteins. Ectopic expression of CD44v6 splice variant confers metastatic potential to non metastatic tumor cell lines, promoting Met activation by its ligand HGF that is mainly secreted by mesenchymal cells [53]. The importance of the CD44v6 and Met multimeric signaling in cancer progression has been strengthened by the observation that adenoma growth in the $Apc^{Min/+}$ mice model was reduced by inhibiting the CD44v6 expression through short hairpin RNA/nanoparticles technology [54]. Moreover, Jung et al. showed that CD44v6 supports tumor cell migration and apoptosis resistance since only the matrix assembled by CD44v6-competent but not-deficient cells induces metastasis formation [55]. Given that disseminating cells are exposed to high levels of ROS during tumor progression, metastatic growth requires also adequate ROS defense ability to successfully colonize secondary sites. Interestingly, knockdown of the redox protein thioredoxin-like 2 has been reported to inhibit tumorigenesis and metastasis of human breast cancer cell lines upon transplantation into immunodeficient mice by enhancing ROS levels and reducing NF-κB activity [56]. It has also been investigated the role of CD44v-xCT in lung metastasis. By promoting xCT-dependent GSH synthesis, CD44 expression allows mouse 4T1 breast cancer cells to evade high levels of ROS produced by neutrophils and colonize the lung. It is not surprising that knockdown of epithelial splicing regulatory protein 1 in $CD44^{+}$ subpopulation induces an isoform switch from CD44v to CD44s, resulting in reduced xCT expression and lung metastasis suppression [57].

Proliferating tumor cells distance themselves from the vasculature and colonize an environment deficient in oxygen and nutrients. Therefore, tumor cells need to reprogram their metabolism by increasing glycolytic activity and decreasing aerobic respiration rate. This shift is mediated by an increase in ROS levels generated by mitochondrial complex III, which accounts for hypoxia-inducible factor-1 (HIF-1) stabilization *via* oxidation/inactivation of prolyl hydroxylases and release from Von Hipper-Lindau (VHL)-mediated degradation. When stabilized in hypoxia, HIF-1α dimerizes with HIF-1β and translocates into the nucleus. By interacting with the co-activators CBP/p300, the α/β heterodimer HIF-1, bound to hypoxia-response elements (HREs) in target genes, mediates the expression of proteins involved in the formation of new vasculature and metabolic adaptation to hypoxia [58]. HIF-1α increases the transcription of glucose transporters and glycolytic enzymes as well as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), resulting in the diversion of pyruvate toward lactate away from mitochondrial oxidative phosphorylation [59]. Additionally, mutations of tumor suppressor genes (*PTEN*, *VHL*) and oncogenic pathways (Ras/MAPK, PI3K-Akt) converge on HIF-1α activation through an oxygen-independent mechanism [58]. Specifically, in CRC hypoxia activation of wild-type K-Ras mediates Akt phosphorylation and resistance to apoptosis [60].

Similar to HIF-1α, HIF-2α is involved in the regulation of hypoxia tumor response. Interestingly, Heddleston et al. reported a role of HIF2α in reprogramming non-stem cancer cells toward a stem-like phenotype by inducing the expression of key stem cell genes, like *OCT4*, *NANOG* and *MYC*. Concordantly, overexpression of HIF-2α in glioma non-stem cells increased neurospheres formation and tumorigenic capacity [61]. Moreover, as shown by Xue et al., HIF2α activation modulates colon tumorigenesis in Apc $^{Min/+}$ mice by overexpression of intestinal iron transport. The resulting iron intake contributes to dysregulation of local iron homeostasis, which in turn affects cancer progression through increasing cell survival and proliferation [62].

Hypoxia has been reported as an important driving force for the multistep process of metastasis. The early EMT-related events induced by hypoxia support ROS-dependent GSK-3β inactivation, followed by SNAIL nuclear translocation and E-cadherin loss [63,64]. In response to hypoxic conditions, Notch signaling up-regulates Snail expression by two distinct but synergistic mechanisms, involving both direct transcriptional activation of *SNAI1* [65] and an indirect mechanism operating *via* the ECM protein lysyl oxidase (LOX) [66]. Moreover, Twist expression, directly induced by HIF-1α through the HRE located in its promoter, contributes to cadherin profile changes with E-cadherin down-regulation followed by N-cadherin upregulation [64]. At a later stage, activation of Wnt/β-catenin pathway and increased invasiveness are sustained by HIF-1α- and VEGF-dependent events [63]. Particularly, hypoxia-induced invasion is associated with basement membrane degradation and ECM remodeling by upregulation of cathepsin D (CTSD) and MMP2 [58,67]. Hongo et al. proposed that the up-regulation of β1 integrin expression by hypoxia in CRC cells increases the ability to adhere and migrate on collagen fibers [68].

The role of HIF-1α in cell migration is related to improved LOX expression. In hypoxic cancer cells, LOX mediates the covalent cross-linking of collagen fibers and elastin, thereby increasing cell focal adhesion kinase activity, known to induce cell motility by acting as a signal between integrins and actin cytoskeleton. These remodeled matrix events are essential for invasive cell movement and provide a metastasis freeway by which other tumor cells may walk and spread to adjacent tissues [69]. Hypoxia-induced "invasive switch" is also mimicked by Met and autocrine motility factor (AMF) overexpression. Pennacchietti et al. demonstrated that hypoxia synergizes with HGF to affect basal cell morphology and induce cell scattering by transcriptional activation of the *MET* proto-oncogene. Consistently, increased Met expression sensitizes tumor cells to HGF produced by fibroblasts, promoting thereby the invasive growth toward tissue parenchyma and blood circulation [70]. One of the most important tumor-secreted cytokines, AMF promotes resistance to apoptosis in tumor cells and angiogenesis induction *via* autocrine and paracrine mechanisms [71].

Hypoxia-selected tumor cells are able to evade the hostile milieu of primary site by promoting angiogenesis and affecting vascular integrity and permeability. Consistently, hypoxia-dependent expression of VEGF, MMP1 and MMP2 is essential to offend the vasculature and promote intravasation. MiR-372/373, upregulated in response to hypoxia through HIF-1α, contribute to increased intravasation by targeting the MMP inhibitory protein RECK, resulting in excessive activation of MMPs [72]. Besides VEGF, MMP1 and MMP2, tumor cells extravasation is promoted by Angiopoietin-like 4 (ANGPTL4), a member of vascular regulators angiopoietin family upregulated in the primary tumor by both TGFβ and hypoxia [58]. As shown by Padua et al., the expression of ANGPTL4 in cancer cells primes these cells to disrupt vascular endothelial tight junctions and increase the capillary permeability, thereby affecting the transendothelial passage [73].

Recent reports suggested that the metastatic seeding at distant organs is influenced by hypoxia-induced factors released from primary tumor, critical for pre-metastatic niche formation. It has been reported that in breast cancer LOX, secreted by hypoxic tumor cells into the bloodstream, modifies the collagen cross-linking in the lungs and promotes the recruitment of $CD11b^+$ myeloid cells to pre-metastatic sites. By the consequent adhesion to cross-linked matrix, $CD11b^+$ myeloid cells produce MMP-2, which supports collagen remodeling by LOX and thereby increases recruitment and subsequent invasion of bone marrow-derived cells. This cell population is thought to create a favorable environment for the incoming primary tumor cells [69].

Hypoxia in primary tumor may also improve metastatic seeding of tumor cells by heightening chemokine C-X-C motif receptor 4 (CXCR4) expression. Specifically, CXCR4-mediated signal transduction can enable tumor cells to home to secondary organs where its ligand Stromal Derived Factor 1 (SDF1) is highly expressed (*e.g.*, lymph nodes, lungs, liver, or bones). The responsiveness of $CXCR4^+$ cells to SDF-1 gradient is positively affected by several molecules produced during inflammation, specifically fibrinogen, fibronectin, C3a, and hyaluronic acid, suggesting that inflammation affects the spreading of $CXCR4^+$ tumor cells [74].

Similarly to primary tumor, hypoxia response molecules facilitate tumor–stromal interactions in secondary sites to support the metastasis colonies proliferation. However, the role of hypoxia in determining the organ-specific metastasis is still unknown. Microarray profiling revealed that hypoxia promotes the expression of lung-metastasis gene signature, including *Connective tissue growth factor*, *Osteopontin*, *IL-6 and -8*, *ANGPTL4*, and primes ER^- breast cancer cells in promoting lung colonization by activating an effective angiogenesis. Since bone marrow vasculature is already fenestrated facilitating the transendothelial passage of tumor cells, hypoxia-induced angiogenesis does not provide an advantage for bone metastasis seeding. Thus, it is not surprising that hypoxia activates a limited percentage of bone-metastasis genes, including *CXCR4* and *dual specificity phosphatase 1*, which functions as a stress-inducible MAPK signaling activator [58,75]. Interestingly, experimental models and human cancers implicated TFGβ in promoting distal metastasis formation. After seeding the lung parenchyma, ER^- breast cancer cells take a proliferative advantage from local TGFβ through induction of the cell differentiation inhibitor ID1 [76]. As shown by Kakonem et al., in mice inoculated by MDA-MB-231 breast cancer cells, osteolytic bone metastases require the recruitment and activation of osteoclasts. In particular,

induction of IL-11 and parathyroid hormone-related protein production by TGFβ promotes differentiation of osteoclast precursors and bone resorption, thereby increasing the osteoblastic expression of Receptor Activator for NF-κB (RANK) ligand [77]. Lastly, Batlle et al. speculated that IL-11, a TGFβ-target gene in stromal cells, confers metastatic initiation capacity to CRC cells *via* GP130/STAT3 signaling, critical to induce a survival advantage and suppress apoptotic stimuli in metastatic sites [78].

5. CSCs and vasculature cells crosstalk: a mutual convenience

Tumor cell growth and nurture require several strategies to supply the oxygen and metabolic demand, all involving new vessels formation and captivation from the surrounding stroma. Tumor neovascularization can occur through (a) sprouting from existing vessels (sprouting angiogenesis), (b) lumen invagination and splitting of vessels (intussusceptive angiogenesis), (c) enfolding of vessels by cancer cells (vessel co-option), (d) simulation of endothelial features by tumor cells (vasculogenic mimicry), (e) formation of lymphatic vessels from pre-existing ones (lymphangigogenesis) and finally (f) endothelial progenitor cells recruitment [79].

Angiogenesis has been defined as a key process for tumor and metastasis formation and CSCs are predicted to be strong promoters of this phenomenon. For instance, Bao et al. demonstrated a profound interplay between CSCs and tumor vasculature. Injection of glioblastoma stem cells (GSCs) $CD133^+$ in the right frontal lobes of athymic nude mice displays strongly angiogenic and hemorrhagic tumors compared to the $CD133^-$ counterpart. The angiogenic advantage of the $CD133^+$ fraction may be supported by a 10–20 fold increase of VEGF secretion. Significantly, conditioned medium from these fractions fosters human endothelial cells migration and tube formation [80]. According to these data, the concomitant presence of CSCs correlates with more angiogenic tumors in terms of enhanced resident endothelial cells function and recruitment of bone marrow-derived endothelial progenitors to the tumor site. VEGF and SDF1 are the main powering determinant of these CSCs properties [81].

On the other hand, it is likely conceivable a possible impact of endothelial cells on CSCs state. A paracrine signaling by endothelial cells may induce CRC cells to gain CSC properties with Notch pathway as the main player of this conversion. Indeed, Jagged-1, a Notch-activating ligand, is released from endothelial cells as a soluble form by ADAM17 proteolitic cleavage and its binding to Notch receptor of adjacent CRC cell triggers the onset of stem-like features. Co-culturing CRC cells either with endothelial cancer cells or with endothelial cell-conditioned medium lead to an increase of the $CD133^+/ALDH^+$ subpopulation compartment and sphere forming capability as well as *in vivo* tumor growth and spreading [82].

Similarly, as showed by Calabrese et al., it was demonstrated that endothelial-derived factors support self-renewing of brain tumor cells and keep them in an undifferentiated state. These stem-like cells closely interact with $CD34^+$ capillaries and are strictly dependent on microvasculature density. Co-injection of primary human endothelial cells and $CD133^+$ medulloblastoma cells accelerates initiation and promotion of brain tumor xenografts by expanding the CSCs pool. Thus, tumor microenvironment orchestrates a vascular niche formation determining the CSCs fate [83].

Furthermore, the presence of 'mosaic' blood vessels in which both endothelial and tumor cells are located into the lumen surface of tumor vessels has long been described [84]. Consistent with these findings, glioblastoma stem cells can be induced to differentiate into endothelial cells and directly contribute to tumor vasculature architecture when injected in immunocompromised mice, as proven by the presence of $CD34^+/CD144^+/VEGFR2^+$ human-derived endothelial cells [85]. Likewise, vasculogenic mimicry can occur *via* a multipotent intermediate ($CD133^+/CD144^+$) that can differentiate either into a tumoral or endothelial phenotype [86].

Another related possibility is that, rather than differentiation into endothelial lineage, CSCs generate vascular pericytes that mainly support endothelial cells to maintain vessels function and integrity. It was recently shown that, after GSC differentiation induction, a fraction of 4–11% cells expressed several pericyte markers such as α-SMA, NG2, CD146 and CD248. Significantly, *in vivo* cell lineage tracing with specific fluorescent reporter confirmed that the majority of pericytes had GSC origin. Of note, selective deletion of GSC-derived pericytes hampered microvessel development and tumor growth. CXCR4 expressing GSCs were recruited toward epithelial cells by an SDF-1 chemoattractant gradient and then induced to pericytes differentiation upon TGF-β release by endothelial cells [87].

6. Angiogenic pathways orchestrate CSCs survival and motility

Although CSCs represent a minority of tumor cells population, deregulation of pathways involved in cell self-renewal and motility contributes to cancer conversion and promotion. In addition to well established CSCs radioresistance and chemoresistance mechanisms, an increasing adaptability to antiangiogenic treatment was shown [88]. These cells can elicit resistance and increase their tumorigenic and invasive potential by exploiting an hypoxic microenvironment [89] as well as the activation of an anti-apoptotic program [88] (Fig. 3).

Among molecules that regulate tumor angiogenesis, such as plateled-derived growth factor (PDGF), FGF, HGF and TGF-α/β, VEGFs and their cognate receptors (VEGFRs) are the driving force of angiogenic response due to their specific expression on endothelial and tumoral cells, resulting in multiple signal pathways activation.

VEGF family is represented by five members (VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor [PGF]) coupled with three tyrosine kinase receptors (VEGFR1 [Flt1], VEGFR2 [KDR/Flk1] and VEGFR3 [Flt4]). As a soluble factor, VEGF serum concentration, in preoperative CRC, reflects the stage and correlates with disease progression. Both VEGFs and VEGFR2 are associated with a worse prognosis, tumor spreading and enhanced microvessel density. Particularly, VEGF expression increases during the colonic adenoma–adenocarcinoma pathogenesis conversion and prior to the invasive phenotype switch [90].

VEGFR1 is mostly expressed on endothelial cells, monocytes, macrophages, hematopoietic stem cells and some tumoral cells, including CRC cells [91]. VEGFB and PGF have been identified as its exclusive ligands. VEGFR2 is not restricted to endothelial cells but it is also shared by, for example, colitis-associated colon cancer epithelial cells [92] and GSCs [93]. Furthermore, VEGFR3, the first normal lymphatic endothelium marker [94], together with VEGFC is involved in cancer lymphangiogenesis [95].

VEGFA/VEGFR2 interaction is recognized as a potent proangiogenic stimulus increasing survival, proliferation, migration, and vascular permeability of endothelial cells [96]. Although VEGFA has a higher binding affinity for VEGFR1, VEGFR2 possesses a greater tyrosine kinases activity that governs the activation of MAP-kinase, PI3K, Fak and Rac pathways. Interestingly, phosphorylation of p38MAPK, in colon CSCs, protects them from antiangiogenic treatment through the activation of Heat shock protein 27 (Hsp27) [88]. Hypoxic induction of VEGF is not merely dependent on HIF-1α. It was already reported that CRC cells are forced to express VEGF through a K-Ras/PI3K/Rho/ROCK/c-Myc axis. Indeed, a putative Myc-Max binding site was found on *VEGF* gene promoter [97].

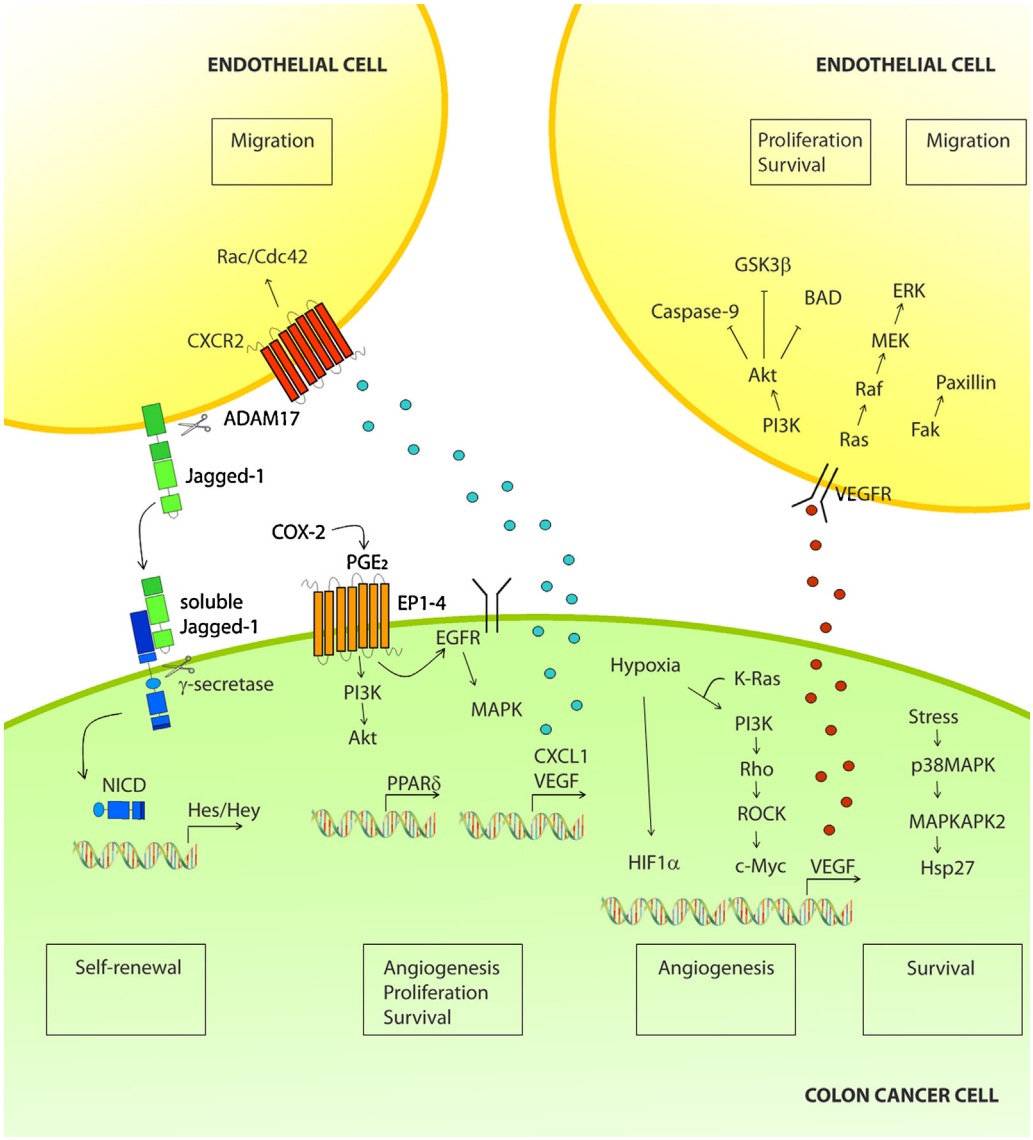

Fig. 3. Tumor microenvironment is conducive to angiogenesis promotion. A truncated soluble form of Jagged-1 is released by endothelial cells and its binding to Notch receptor on nearby colon cancer cells promotes a stem-like phenotype. PGE_2 mediates the release of the angiogenic factors CXCL1 and VEGF in colon cancer cells, *via* an EP1-4/EGFR/MAPK cascade. CXCL1 secretion stimulates endothelial cell migration by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE_2 induces colon cancer cell proliferation and survival trough PI3K/Akt signaling and transcriptional activation of PPARδ. Under hypoxic conditions, induction of HIF1α and alternative K-Ras pathways results in further VEGF release from cancer cells. In endothelial cells, VEGF/VEGFR interaction promotes cell proliferation, survival and migration *via* PI3K, Ras and FAK pathways. Finally, activation of pro-survival signals in tumoral cells is triggered by microenvironmental stress and p38MAPK, MAPKAPK2 and Hsp27 cascade. Notch intracellular domain (NICD), prostaglandin E2 (PGE_2), chemochine C-X-C motif ligand 1 (CXCL1), prostaglandin E receptor 1–4 (EP1-4), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), chemochine C-X-C motif receptor 2 (CXCR2), cell division control protein 42 (Cdc42), perixisome proliferator-activated receptor δ (PPARδ), Rho-associated protein kinase (ROCK), MAP kinase-activated protein kinase 2 (MAPKAPK2), Heat shock protein 27 (Hsp27).

It was extensively observed that Prostaglandin E2 (PGE_2) is abundantly secreted by both colon cancer cells and stromal cells and promotes the release of the angiogenic factors C-X-C motif ligand 1 (CXCL1) and VEGF through the Prostaglandin E receptor 1–4 (EP1-4)/Epidermal growth factor receptor (EGFR)/MAPK cascade. Tumor-derived CXCL1 stimulates endothelial cell migration and *in vivo* tumor growth and microvessels density by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE_2, *via* PI3K/Akt signaling, enhances transcriptional activation of Perixisome proliferator-activated receptor δ (PPARδ) required for colorectal adenoma growth [98,99].

The angiogenic properties of VEGF may be amplified when tumoral endothelium is previously destabilized by other growth factors, such as Ang-2. Ang-1, 2 and 4 bind the same endothelial receptor Tie2. While Ang-1 is expressed by pericytes, smooth muscle cells and tumor cells, Ang-2 is exclusive to endothelial cells. Ang1 preserves vascular integrity by reducing cell-to-cell gaps whereas Ang2 increases pericytes dissociation and vessels destabilization, rendering endothelial cells more receptive to foreign stimuli, for instance, VEGF [100].

A broad spectrum of clinical data reports that activating *KRAS* mutations could occur up to 50% of early stages CRC patients [101]. Interaction of Ras with the catalytic subunit p110 of PI3K appears to be extremely relevant to the induction of *VEGF* gene expression. PI3K phosphorylates Akt, which subsequently inhibits GSK-3β leading to β-catenin nuclear translocation. Mutated *KRAS* enhances the stability of β-catenin and promotes the formation of nuclear β-catenin/TCF4 complexes [102]. In addition, further evidence of a cooperative interaction between K-Ras and Wnt pathway in CRC lies in the presence of a consensus TCF4 element in the *VEGF* promoter [103]. At the early onset of colon neoplastic lesion, a crosstalk between Ras and the microenvironment has been described.

Particularly, *RAS* oncogene can orchestrate endothelial and inflammatory cells recruitment to the tumor site in an IL-8-dependent manner [104]. On the other hand, as previously mentioned, in wild type *KRAS* CRC and in presence of an hypoxic microenvironment, VEGF expression is strictly regulated by Akt and c-Src pathways [60].

Entirely conflicting with other Ras oncoprotein features, R-Ras is described as a supporter of tumor vessels normalization by counteracting VEGF angiogenic potential. Tumor vasculature differs from the normal counterpart for the presence of saccular, tortuous and high permeable vessels with fibrin-gel matrix deposition. Pericytes are poorly associated with endothelial cells supported by an irregular basement membrane. Vessel leakiness allows cancer cells to easily penetrate into the bloodstream and thus colonize distant organs. In addition, plasma leakage from vessels, due to an higher interstitial hydrostatic pressure at the tumor site, reduces the delivery of chemotherapeutic agent [105]. However, R-Ras does not affect the oxygen-sensing mechanism of vessel normalization exerted by PHD2 or HIF-2α under hypoxic condition. Conversely, it facilitates the accumulation of VE-cadherin on cell-to-cell junction, favoring the stabilization of the endothelial barrier. Indeed, it reduces phosphorylation of Ser665 in the cytoplasmic domain of VE-cadherin, suppressing its internalization on endothelial cells. Interestingly, this phenomenon antagonizes VEGF-mediated VE-cadherin phosphorylation. Furthermore, R-Ras activity in pericytes increases their interaction with endothelial cells, leading to normal vessels morphogenesis [106].

Based on this observation, antiangiogenic therapies may contribute to the normalization of tumor vasculature architecture and consequently improve their distribution and efficacy [107].

Finally, the BMPs pathway was observed aberrantly regulated in the majority of sporadic CRC and germline mutation on BMP receptors and downstream substrates were detected in juvenile polyposis [108]. Furthermore, BMP signaling has been shown to be essential in human intestinal development and regeneration regulating also the number and the self-renewal state of colonic stem cells [109]. To date, little is known about BMPs role in angiogenesis. Recently, BMP9 was identified as a ligand of the orphan Activin receptor-like Kinase 1 (Alk1) in endothelial cells and the resulting interaction affects several angiogenic steps. BMP9/Alk1 signaling counteracts bFGF-stimulated endothelial cells proliferation and migration as well as VEGF-induced angiogenesis. Indeed, BMP9/Alk1/BMP receptor II (BMPRII) complex abolished VEGF expression through suppression of TGFβ/Alk5/BMPRII signaling [110]. Certainly, further investigations are needed to identify the underlying mechanism of BMP engagement during angiogenesis promotion.

7. Therapeutic advances

Quiescent cells within the stemness niche have been associated with tumor recurrence and relapse after chemotherapy. Targeting the molecular mediators and signaling pathways affecting EMT and tumor progression may provide novel therapeutic strategies to prevent CSCs-dependent distant metastasis formation.

Fighting neovascularization to counteract cancer promotion is a crucial step of the long-standing theory of Folkman [111]. Based on this hypothesis, the first antiangiogenic compound approved by the FDA, in 2004, was Bevacizumab. It is a monoclonal antibody against VEGF recommended in first and second line settings, either with FOLFOX (5-Fluorouracil, Leucovorin and Oxaliplatin) or FOLFIRI (5-Fluorouracil, Leucovorin and Irinotecan). As shown by preclinical data, Aflibercept is a VEGFA, VEGFB and Placental growth factor (PIGF) decoy receptor, composed of VEGFR1 and VEGFR2 extracellular domains fused to the constant portion of immunoglobulin gamma chain. In 2012, FDA approved the administration of this compound plus FOLFIRI in patients with metastatic CRC with disease progression after oxaliplatin treatment. Recently, advanced clinical trials validate the efficacy of Regorafenib as a VEGFR1/2/3 and Tie2 tyrosine kinase inhibitor [112].

Despite initial therapeutic benefits in patients with metastatic CRC, classic antiangiogenic strategies failed to improve long-term clinical outcomes [113].

Since new development of tumor vasculature implies several complex signaling, alternative angiogenic or anti-apoptotic mechanism could be devised by cancerous cells [88]. Indeed, it has been recently pointed out, by Lu et al., that glioblastoma multiforme treatment with Bevacizumab developed more invasive tumors, as the blockade of VEGF enhances HGF-induced MET phosphorylation [114]. Another attractive approach takes into account that anti-angiogenic treatments favor a hypoxic microenvironment that gives to CSCs population a metabolic advantage and preserves their self-renewal state [89].

Given that anti-angiogenic drugs may enhance tumor invasiveness by blocking *de novo* angiogenesis and inducing hypoxia, the development of HIF-1α targeted therapies may reduce or prevent metastasis [58]. There are several agents that affect directly or indirectly the HIF-1α expression or activity. The binding of HIF-1α to the co-activator p300/CBP has been attenuated by the chetomin, a small molecule that interferes with hypoxia-inducible transcription [115]. In addition, the proteasome inhibitor bortezomib, approved for treatment of patients with multiple myeloma and mantle cell lymphoma, impairs the interaction with the co-activator p300/CBP by inducing the hydroxylation of Asn803 in the C-terminal transactivation domain [116]. By blocking HIF-1α binding to HRE sequence, a step required for transcription induction, anthracyclines have been reported to significantly reduce the prostate tumor growth and vascularization in a mouse model [117]. The topoisomerase I inhibitor topotecan, cardiac glycoside digoxin and PX-478 have also been implicated in HIF-1α expression, consistent with their remarkable antitumor activity in a variety of human tumor xenograft models [118]. HIF-1α protein translation is also inhibited by the chaperone Hsp90, which induces its proteasomal degradation in a VHL-independent manner [119]. Nontoxic prodrugs that generate active species in hypoxic tissue by selective bioreduction have now reached advanced clinical trials. Nitroaromatics, quinones, tertiary amine N-oxides, and transition metals are selectively reduced and activated in the absent of O_2 to release or activate toxic effectors to eradicate surrounding hypoxic tumor cells. Similarly, the gene-directed enzyme prodrug therapy uses HRE sequence to improve the expression of reductase enzymes, including P450 reductase, HSV thymidine kinase and cytosine deaminase, which kill hypoxic tumor cells by converting a prodrug into a cytotoxin [58]. Nevertheless, a robust validation of hypoxia inhibitors in clinical trials is needed to support the hypoxia-targeted therapies. Overall, these findings suggest that advanced compounds need to be developed to selectively target cancer microenvironment.

8. Conclusions

The reviewed data emphasize the supporting role of the microenvironment in primary tumor establishment and dissemination to distant sites. The critical event of EMT depends on the complex signals produced by stromal components ensuring the generation of CSCs phenotype with increased proliferative capacity and metastatic potential in hostile milieu. In addition, perivascular, hypoxic and premetastatic niches have been proposed to enhance the resistance of CSCs to therapy. Based on this observation, combination therapies targeting hypoxia and *de novo* angiogenesis may have enormous therapeutic implications by blocking the successful homing of cancer cells to metastatic sites. Thus, a better

understanding of cancer microenvironment framework could be a crucial key to improving patient cure.

Conflicts of interest

None declared.

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